PERSPECTIVE

Iron, Oxidative Stress and the Example of Solar Ultraviolet A Radiation†

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Abstract

Iron has outstanding biological importance as it is required for a wide variety of essential cellular processes and, as such, is a vital nutrient. The element holds this central position by virtue of its facile redox chemistry and the high affinity of both redox states (iron II and iron III) for oxygen. These same properties also render iron toxic when its redox-active chelatable 'labile' form exceeds the normal binding capacity of the cell. Indeed in contrast to iron bound to proteins, the intracellular labile iron (LI) can be potentially toxic especially in the presence of reactive oxygen species (ROS), as it can lead to catalytic formation of oxygen-derived free radicals such as hydroxyl radical that ultimately overwhelm the cellular antioxidant defense mechanisms and lead to cell damage. While intracellular iron homeostasis and body iron balance are tightly regulated to minimise the presence of potentially toxic LI, under conditions of oxidative stress and certain pathologies, iron homeostasis is severely altered. This alteration manifests itself in several ways, one of which is an increase in the intracellular level of potentially harmful LI. For example acute exposure of skin cells to ultraviolet A (UVA, 320-400 nm), the oxidising component of sunlight provokes an immediate increase in the available pool of intracellular LI that appears to play a key role in the increased susceptibility of skin cells to UVA-mediated oxidative membrane damage and necrotic cell death. The main purpose of this overview is to bring together some of the new findings related to intracellular LI distribution and trafficking under physiological and patho-physiological conditions as well as to discuss mechanisms and consequences of oxidant-induced alterations in the intracellular pool of LI, as exemplified by UVA radiation.

Key words: Iron, labile iron pool, oxidative stress, reactive oxygen species, skin, UVA

1 Introduction

1.1 Iron and Oxidative Stress

Iron is the second most abundant metal, after aluminium, and the fourth most abundant element in the earth's crust (5%). Living organisms from bacteria to mammals use iron for vital biological processes, as part of, or as a cofactor of proteins and enzymes.[1] Iron plays a key role in mammalian cells' growth, respiration and replication. Many iron-containing proteins catalyze key reactions involved in energy metabolism (cytochromes, mitochondrial aconitase, iron-sulfur proteins of the electron transport chain), respiration (e.g. hemoglobin), and DNA synthesis (i.e. ribonucleotide reductase) and it is well known that iron depletion leads to G1/S cell cycle arrest and apoptosis.[2] Additionally, iron-containing proteins are required for the metabolism of collagen, tyrosine and catecholamines.[3]

Iron is a transition metal that can exist in two stable configurations: electron donor ferrous (Fe^{2+}) and electron acceptor ferric (Fe^{3+}). The easy access to two oxidation states allows iron to act as a catalyst in mammalian cellular pathways that involve redox mechanisms, however this same property makes iron toxic when its redox-active chelatable 'labile' form exceeds the normal binding capacity of the organism.[4] Indeed in contrast to iron bound to proteins, the intracellular labile iron (LI) can undergo redox cycling between its most stable oxidation states Fe^{+2}/Fe^{+3} and react with ROS such as superoxide anion ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) giving rise to hydroxyl radical ($OH^{\bullet}$) via the Fenton reaction (equation **2**) or via superoxide-driven Fenton chemistry (equation **3**).[5] Such reactive ROS are capable of interacting with most biomolecules, depending on the site of bound iron, including sugars, lipids, proteins, and nucleic acids. These interactions promote various harmful processes in cells including lipid peroxidation, protein oxidation, DNA/RNA oxidation and DNA lesions and ultimately overwhelm the cellular antioxidant defense mechanisms and lead to cell damage and death.

$$O_2^{\bullet-} + Fe^{3+} \longrightarrow Fe^{2+} + O_2 \qquad \textbf{(1)}$$

$$H_2O_2 + Fe^{2+} \longrightarrow OH^{\bullet} + OH^{-} + Fe^{3+} \qquad \textbf{(2) (Fenton reaction)}$$

Net: $$O_2^{\bullet-} + H_2O_2 \xrightarrow{\text{Iron}} OH^{\bullet} + OH^{-} + O_2 \qquad \textbf{(3)}$$

1.2 UVA and Oxidative Stress

At the cellular level, the interaction of solar ultraviolet (UV) radiation with biological material changes as a function of wavelength and requires the absorption of the radiation by biomolecules. The UVB (290-320 nm) region of sunlight overlaps with the DNA absorption spectrum and as a result, the direct absorption of UVB by cellular DNA causes DNA photodamage and mutagenesis.[6] In contrast, the UVA (320-400 nm) region of sunlight is weakly absorbed by most biomolecules but is predominantly oxidative in nature, generating ROS via photochemical interactions with intracellular chromophores.[7] The ROS generated by UVA are certainly involved in cytotoxicity, as it has been shown that UVA inactivation of mammalian cells is strongly oxygen-dependent.[8] Furthermore there is evidence from *in vitro* studies that UVA irradiation of macromolecules can cause the generation of H_2O_2 and $O_2^{\bullet-}$ and that iron-catalyzed reduction of H_2O_2 by $O_2^{\bullet-}$ can further generate the highly reactive $OH^{\bullet}$.[9,10] Studies in prokaryotic and eukaryotic cells indicate that ROS may also be generated *in vivo* by UVA irradiation.[7,9,11] UVA may also trigger the formation of ROS long after UVA exposure via non-photosensitised mechanisms. This may occur by activation of enzymatic systems such as NADPH oxidase that generates $O_2^{\bullet-}$.[12] Based on such considerations, the UVA component of sunlight is now considered as a generator of intracellular oxidative stress.

The uncontrolled production of ROS by UVA is undesirable and thus a number of protective strategies are adopted by cells to prevent their formation. Mammalian cells possess several antioxidant enzymes including superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx). In addition, tissues contain many antioxidant molecules such as the endogenous compound glutathione (GSH), which is a major contributor to cellular reducing equivalents and compounds derived from the diet such as vitamins (e.g. alpha-tocopherol, ascorbate), carotenoids and flavonoids. It has been shown that GSH plays a critical role in cellular defence against the lethal action of both UVA and UVB radiations.[13] All these antioxidant enzymes and molecules would be expected to be important in cellular defence against UVA-induced oxidative stress. However the release of LI in skin cells that occurs immediately after UVA irradiation[14] provokes the excess production of highly reactive ROS such as $OH^{\bullet}$ which can overwhelm the antioxidant capacity of the cells leading to cell damage and possible death (see sections **1.3** and **2.3**).

1.3 UVA, Iron and Oxidative Stress

Under normal conditions, iron levels in cells and body are under extremely tight control and there is little opportunity for iron-catalysed free radical generating reactions to occur. However upon exposure to oxidising agents such as UVA, the intracellular iron status changes dramatically as UVA leads to both LI and free heme releases in cells.[14,15] Although heme itself is not a source of LI, it is a substrate for heme-catabolising enzyme heme oxygenase (HO) which could release the heme iron and expand the pool of potentially harmful cytosolic LI.[16] The dual role of UVA as ROS generator and LI enhancer classifies this radiation as a potent oxidising component of sunlight. Indeed UVA-mediated generation of ROS not only induces direct oxidative damage to skin constituents but also leads to an immediate increase in cytosolic labile iron pool (LIP) which in turn exacerbates the oxidative damage already occurring in the skin cells. Both singlet oxygen ($^1O_2$) and H_2O_2, the most important ROS species generated by UVA, exert their biological damage in skin cells predominantly via iron-catalysed oxidative reactions.[17-20] For example it has been shown that physiologically relevant doses of UVA induce lipid peroxidation in membranes of human primary fibroblasts and keratinocytes via pathways involving iron, H_2O_2 and $^1O_2$.[17,21,22] In fact lipid peroxidation that is considered as the prominent marker of UVA-induced photo-oxidative damage in skin involves iron as the major contributor for initiation and propagation of peroxidative damage.[17,21-23] This is because iron 'at' or 'near' strategic targets such as cell membranes, can undergo redox cycling by reacting sequentially with one electron reductants and oxidants, thereby generating toxic oxidants such as $OH^{\bullet}$ and lipid derived alkoxyl and peroxyl radicals and can elicit biological damage.[5,24] Lipid peroxidation results in the destruction of membrane function and structure, disturbed membrane fluidity and ultimately loss of membrane integrity and cell lysis.[25]

The UVA-induced expansion of intracellular LIP ultimately overwhelms the cellular antioxidant defense mechanisms and leads to cell damage.[26] Depending on the severity of the UVA dose applied, the LI-mediated oxidative damage may lead to cell death by apoptosis or necrosis.[27,28] Alternatively the damage may be processed in such way that the carcinogenesis process is initiated or promoted.[26] The injurious consequences of iron-catalysed damage exerted by UVA have been shown to play a key role in skin photoaging and the promotion of skin cancer.[23,26,28,29]

The understanding of the mechanisms underlying the disturbance of iron homeostasis by UVA appears therefore crucial to understand how UVA interacts with cells and tissues.

Here we first provide an overview of iron distribution in cells and body and then introduce the iron homeostasis machinery composed of a complex network of transporters, storage molecules and regulators that coordinately govern iron absorption, iron recycling, and the mobilisation of stored iron in the organism. While these regulatory networks minimise the pool of potentially toxic LIP in cells and tissues, we describe how iron homeostasis is altered under oxidative and pathological conditions. Using this knowledge, we then discuss the mechanisms and consequences of oxidant-induced alterations in the intracellular pool of LI, as exemplified by UVA radiation.

2 Iron Absorption, Storage, Transport and Distribution

2.1 The Body Iron Pool

Iron is absorbed from the mammalian gastrointestinal tract by two protein-mediated mechanisms, one absorbing iron as Fe^{2+} and the other iron as heme.[30] Primates have evolved not to be able to excrete iron, and therefore body iron levels are totally controlled by the absorption process.[31] The total amount of iron in an average human body is about 4-5 g, the majority (i.e. 75%) of which is incorporated into the heme complex and can be found in proteins such as hemoglobin and myoglobin.[32] Only a very small amount of the total body iron is present as an essential component of a very large number of heme (e.g. cytochromes, catalase, oxidases and peroxidases) and non-heme proteins and enzymes (e.g. ribonucleotide reductase and iron-sulfur proteins). All of these may account for no more than 10% of the total body iron pool. The remaining 15% can be found principally in the cytoplasm in the form of the iron storage protein ferritin (Ft) but also in lysosomes, as hemosiderin.

Storage iron is found in the liver, mainly in the hepatocytes, but it is also found in macrophages in the liver, bone marrow, spleen and muscles, where it is readily available as a reserve in case of blood loss.[33,34] Due to epithelial shedding in the gastrointestinal tract and the skin and because of blood loss in women, there is an average daily loss of 1-2 mg. This loss is usually compensated for by the absorption of iron through the diet which contains approximately 10-20 mg of iron of which 1-2 mg is absorbed under normal circumstances. This absorption is increased several-fold when iron levels are low (e.g. in anaemia and hypoxia) and decreased when the iron stores are replete (e.g. in iron-overload conditions and inflammation).[31,35]

2.2 The Labile Iron Pool

All the iron-complexing molecules leave body fluids and cells with an extremely low concentration of free transit iron. Nevertheless there is now strong evidence for the existence of a transit pool of catalytically active iron complexes which is distinct from intracellular iron associated with proteins and is known as the labile iron pool (LIP). Iron belonging to this pool is considered to be in steady-state equilibrium, loosely bound to low-molecular-weight compounds, accessible to permeant chelators and metabolically and catalytically reactive.[36] The LIP has been detected both in the systemic circulation and inside cells and is likely that all daily uptake of the dietary iron (1-2 mg) will pass via the LIP stage before it is sequestered by target proteins.[37]

The LIP is associated with important functions: (a) physiologically, as readily available source of iron for incorporation into proteins; (b) pharmacologically, as target for chelators or metal scavengers; and (c) toxicologically, as vehicle for promoting the formation of free radicals.[38]

2.2.1 The Intracellular Labile Iron Pool. The continuous demand of iron for cellular function is thought to force a permanent flux of LI from the extracellular milieu to the cytoplasm. The cellular LIP in quiescent conditions comprises only minor fractions of the total cellular iron (i.e. less than 5%).[4] Cabantchik and co-workers have defined the intracellular LIP operationally as a cell chelatable pool that comprises both ionic forms of iron (Fe^{2+} and Fe^{3+}) associated with a diverse population of ligands such as organic anions (phosphates and carboxylates), polypeptides, and surface components of membranes (e.g. phospholipid head groups).[4,37] This definition implies that LIP can not only potentially participate in redox cycling but also be scavenged by permeant chelators. The latter property forms the basis for the quantification of the cellular LIP. [4,37]

2.2.2 The extracellular Labile Iron Pool. In contrast to intracellular LIP, the presence of extracellular LIP is often associated with pathological conditions. The extracellular LIP (also called labile plasma iron) has been originally observed in iron overload β-thalassemia patients whose plasma transferrin (Tf) iron-binding capacity has been exceeded.[39] Further to β-thalassemia, other conditions of iron imbalance have been defined

(i.e. hemochromatosis), in which the extracellular LIP has been found to be bound to ligands other than Tf as non-Tf-bound iron.

2.3 Iron Homeostasis

To minimise damage caused by LI, both plasma and intracellular LIP are tightly regulated by means of a complex network of transporters, storage molecules and regulators that coordinately govern iron absorption, iron recycling, and the mobilisation of stored iron. These regulatory networks maintain an adequate level of iron for vital cell, tissue and organ functions, while also minimising the pool of potentially toxic LIP.

The protein central for systemic iron homeostasis is the hepatic peptide hormone hepcidin that regulates plasma iron concentrations and tissue iron distribution by inhibiting dietary iron absorption and mobilization. Hepcidin acts by controlling the number of iron channels through which cellular iron is delivered into plasma.[40] In the small intestine, iron is transported out of the enterocyte into the blood via the basolateral iron export protein, ferroportin-1 (FP1).[41,42] When plasma iron is in excess, hepcidin binds to FP1 receptor. This leads to internalisation and degradation of the FP1 receptor which in turn cause the blockage of iron efflux into plasma, resulting in iron retention within cells.[40] Hepcidin follows an inverse relationship with body iron levels: low levels can potentially cause iron overload, whilst high levels cause anaemia.[35,40] In turn, hepcidin concentration is regulated by a variety of influences. Iron loading increases hepcidin production (to block further iron absorption and maintain homeostasis), and so does inflammation (to decrease extracellular iron concentration and its accessibility to micro-organisms).[43]

Alterations in intracellular LIP are normally sensed by the cytosolic iron regulatory proteins 1 and 2 (IRPs) which function as post-transcriptional regulators of both iron uptake via the transferrin receptor 1 (TfR1) and iron sequestration by the iron storage protein, Ft. This regulation occurs via binding of IRPs to conserved iron responsive elements (IRE) in the untranslated region of Ft and TfR1 mRNAs.[44] When iron is scarce in the LIP, Ft and TfR1 mRNAs are specifically recognized and bound by the active forms of IRPs, leading to stabilisation of the TfR1 mRNA and inhibition of Ft translation, both of which will lead to enhanced levels of LIP. Conversely, during an increase in iron supply, IRPs are converted to low affinity mRNA-binding proteins, leading to the induction of Ft mRNA translation and the degradation of TfR1 mRNA, which will ultimately lead to a reduction in the LIP.[44]

Iron regulatory protein 1 (IRP1) is the cytoplasmic counterpart of mitochondrial aconitase, the enzyme which converts citrate to isocitrate through a cis-aconitate intermediate in the tricarboxylic acid cycle by virtue of a catalytic iron-sulfur cluster.[1] In iron-replete cells the cluster is assembled and IRP1 displays aconitase activity; in iron-depleted cells the cluster is lacking and IRP1 functions as an mRNA-binding protein. This reversible switch between a cluster-containing holoprotein and a cluster-deficient apoprotein therefore allows aconitase/IRP1 to constantly sense LI levels and to adapt them to cell requirements. Iron regulatory protein 2 (IRP2) is highly homologous to IRP1 but lacks aconitase activity, probably because of its inability to assemble an iron-sulfur cluster; the protein accumulates in iron-deficient cells and is rapidly targeted for proteasomal degradation in iron-replete cells.[1] Unlike IRP1, IRP2 has a characteristic pattern of tissue expression[44] and is also more sensitive to variations of iron in the diet.[1] Moreover, when abundantly or uniquely expressed, it can act as the major or only modulator of intracellular iron metabolism.[1]

2.3.1 Iron Homeostasis and Pathologies. Disruption of iron homeostasis in cells and body causes a severe unbalance in the availability of potentially harmful LI leading to a variety of disorders associated with iron-deficiency or -overload.[40,45,46] Iron-overload conditions may occur either locally as in ischemic tissue or systemically as with genetic hemochromatosis or transfusion-induced iron overload. In such circumstances elevated levels of LI ultimately lead to free radical-mediated tissue/organ damage.[47]

The involvement of H_2O_2 in numerous types of cell and tissue injury is well-documented.[48-51] Although H_2O_2 itself has low reactivity towards cell constituents, it is capable of forming highly reactive ROS in the presence of trace amounts of catalytic LI via Fenton reaction. Under physiological conditions, cells protect themselves either by the H_2O_2 –degrading enzymes catalase and GPx,[52] or by minimising the intracellular level of potentially harmful LIP via the cytosolic IRP1 and IRP2 regulatory network.[44,53] However under pathological conditions, these conventional cellular defences are often insufficient, because the system is either overwhelmed by an increased H_2O_2 formation[49,54-56] and/or by an excess presence of LI.[57] The simultaneous presence of excess redox active chelatable LI and H_2O_2 can be potentially toxic for cells as it can catalyse the formation of oxygen free radicals such as $OH^{\bullet}$ and can elicit irreversible oxidative damage. The pathological consequences of iron-catalyzed oxidative damage are recognized in diseases such as hepatitis, liver cirrhosis, hemochromatosis, cancer, chronic inflammatory disorders and neurodegenerative diseases.[58-

[66] Excess iron may also aggravate diabetes, cancer, cardiovascular disease and alcoholic and non-alcoholic steatohepatitis.[67-71]

High levels of LI have also been identified as a risk factor for the development of cancer.[72] Numerous studies across a variety of populations have found a positive correlation between iron stores in the body and risk of the development of a range of cancers including colorectal, liver, kidney, lung and stomach cancers.[73] High dietary iron has also been reported to induce skin cancer in mice.[74] Furthermore in both animals and humans, primary neoplasms develop at body sites of large iron deposits such as skin, which is a potential target for significant oxidative damage due to its constant exposure to high oxygen tensions and frequent exposure to ultraviolet (UV) light.[32] During the process of carcinogenesis, intracellular iron homeostasis is altered, with neoplastic cells, poor in iron relative to their normal counterparts, internalizing iron from Tf at a tremendous rate, despite low levels of the iron storage protein, Ft.[2]

The presence of excess iron has also been demonstrated in a variety of skin disorders such as psoriasis,[75] venous ulceration[76] and atopic eczema,[77] indicating the involvement of iron in the pathology of skin.

Iron accumulation in the body also occurs with age in males. In women however, iron accumulation does not occur until after the menopause. Iron accumulation in post-menopausal women has been linked to increased incidence of heart disease.[78] Increased levels of iron in post-menopausal women have also recently been considered as etiological agents potentially involved in the increased risk of oxidative damage in skin.[79] There is also evidence for age-related accumulation of LIP associated with rheumatoid arthritis, atherosclerosis and Alzheimer's disease (AD).[68] Interaction of iron and cholesterol in promoting oxidative damage has been suggested as causative of both atherosclerosis and neurodegeneration.[80] Mutations in genes involved in controlling iron have also been shown to cause predisposition to AD.[68] Iron may also play a role in the carcinogenic process of other transition metals such as copper, or other types of carcinogens.[72]

2.3.2 Iron Homeostasis and Oxidative Stress. In addition to pathological conditions, iron homeostasis can also be severely altered under oxidative stress conditions. For example, IRP1 is susceptible to oxidative inactivation of RNA binding *in vitro* and *in vivo*.[81,82] By contrast, menadione, nitric oxide, and H_2O_2 activate IRP1 RNA-binding activity

and, as a result, lead to a coordinated decrease of Ft synthesis and induction of TfR1 expression.[83,84] On the other hand, Cairo *et al* have observed that in cell-free systems, H_2O_2 cannot directly modify IRP1 but instead can cooperate with $O_2^{\bullet-}$ and down-regulate IRP1 activity.[85] The authors have further demonstrated that down-regulation of IRP1 occurs also *in vivo* in liver tissue of rats subjected to ischemia reperfusion or phorone, a GSH-depleting agent.[57,86]

We have also observed that exposure of skin cells to UVA (320-400 nm) inactivated the mRNA-binding activity of IRP1[14] and that this correlated with a reciprocal increase in the aconitase activity of the IRP-1 (which is known to occur in response to the increased availability of intracellular LIP[1]). The inactivation of IRP1 by UVA in skin fibroblasts is related to the excess LI and this was confirmed by measuring the LIP levels with the fluorescent Calcein assay.[36] From these observations, it was concluded that UVA induces immediate release of LI in the skin fibroblast cells.[14] Further studies from this laboratory revealed that in addition to UVA, H_2O_2 treatment of skin fibroblasts also promotes a dose-dependent increase in intracellular LIP.[26,87,88] Furthermore our investigations revealed that the proteolytic degradation of Ft by UVA is a major contributor to the observed increase in LI in irradiated skin fibroblasts.[14] Numerous other studies have also demonstrated that pro-oxidant stresses are capable of modulating iron homeostasis by elevating the intracellular LIP via release of iron from sensitive sites, such as iron-sulfur clusters and Ft. Stress stimuli for which this has been observed include peroxides, nitrofurantoin, nitric oxide, phorone, ischemia-reperfusion and hypothermia injury.[14,26,37,57,86,89]

In addition to IRP1, IRP2 is also specifically modulated in response to oxidative stress in the form of enhanced production of ROS and reactive nitrogen intermediates.[1]

Oxidative stress can also affect iron homeostasis via activation of heme oxygenase 1 (HO-1). The strong transcriptional activation of HO-1 by UVA was first identified in human skin fibroblasts.[90] Later studies demonstrated that this phenomenon also occurs in melanocytic and dendritic cells, and in certain pathologic states it is detected in epidermal keratinocytes.[91] It is now recognised that all conditions that lead to perturbation of heme homeostasis (e.g. oxidative stress, tissue injury and inflammation) lead to rapid changes in heme synthesis (via aminolevulinate synthases) and catabolism (via HO). This adaptive response to restore heme homeostasis in oxidative stress and related pathological conditions must also take care of any perturbations in cellular LIP so that Ft is almost always induced, albeit more slowly, under the same conditions. The aberrant cellular and tissue iron

distribution and severe iron-deficient anemias observed in HO-deficient rodents (and in the one human case recognised to date) indicate that heme oxygenases themselves also play a crucial role in controlling tissue iron distribution and homeostasis.[92]

Studies from Tyrrell's laboratory have shown that acute exposure to UVA or H_2O_2 promotes immediate heme release from microsomal hemoproteins in a human primary skin fibroblasts cell line, FEK4.[15] Although heme itself is not a source of LIP, it is a substrate for the heme-catabolizing enzyme, HO which could release the heme iron. In the short term, HO activity can cause hypersensitivity to oxidative UVA radiation due to release of iron from heme.[93] In the long term (i.e. 1-2 days) however, the UVA-mediated activation of HO-1 leads to HO-1-dependent increase in Ft[94] and a consequent lowering of the pro-oxidant state in skin cells.[95] Studies from Girotti and co-workers have also shown that short-term treatment of murine lymphocytic leukemic cells with hemin, causes an early increase in HO-1 and LI that sensitises the cells to acute oxidative killing, however long-term hemin treatment results in elevated H-Ft levels that sequesters the potentially harmful LI and enhances cellular resistance to acute exposure to oxidants.[96] In contrast to acute conditions, repeated exposure of cells to oxidizing agents such as UVA or H_2O_2 has been shown to induce the HO-1 refractory response presumably as a safety mechanism to shut off the excess LI production as a result of repeated heme breakdown.[16,97-99]

Recent research aimed at understanding the mechanisms underlying the oxidant-induced alterations in iron homeostasis has revealed that the differential subcellular LI distribution plays a key role in promotion of iron-catalysed oxidative damage in cells. Furthermore translocation and trafficking of LI between subcellular compartments appears to be a hallmark of oxidative stress leading to propagation of iron-catalysed oxidative damage in cells. In order to understand the consequences of alteration of iron homeostasis by UVA, it is therefore necessary to understand how LI is distributed in subcellular compartments under physiological and oxidative conditions. The following sections provide an overview of the recent findings in this domain.

2.4 Labile Iron Distribution in Subcellular Compartments

Due to the high physiological and patho-physiological importance of LI distribution, great efforts have been made to develop suitable methods to investigate the intra- and sub-cellular LI levels. Most methods to characterize intracellular LI distribution seriously perturb the

extent and nature of the LIP, rendering them inapplicable to living cells.[100] In combination with non-invasive and high resolution confocal imaging, fluorescent chemosensors have proven to be highly sensitive tools to investigate the metabolism of LIP in individual cells with subcellular resolution. The methodology developed by Cabantchik and co-workers using Calcein-AM, a fluorescein derivative has so far been the most frequently applied fluorescent chemosensor to study intracellular LIP.[4] However, due to hydrophilic nature of Calcein (CA) and lack of access to subcellular compartments, it has been demonstrated that the CA-based measurements reflect mostly the cytosolic LI rather than the overall intracellular LIP.[101] Using this methodology, we observed that the cytosolic LIP levels of primary human skin keratinocytes were 2-3 fold lower than their matched primary human skin fibroblasts.[28] The lower cytosolic LI content of skin keratinocytes correlated with their higher resistance to UVA-induced oxidative damage.[28] Interestingly CA-assay also revealed that the cytosolic LIP content of highly proliferative human keratinocytes is much higher (i.e. 3-4 fold) than the low proliferating primary skin keratinocytes, as observed in a series of squameous cell carcinoma and psoriatic cell lines isolated from patients (unpublished data, this laboratory). The high requirement of neoplastic cells for iron that is required for rapid cell division has been shown to provoke an adaptive cellular response in the form of high Tf-bound iron uptake by increased TfR1 expression and low iron storage by decreased Ft content to maximise the availability of intracellular LIP for ribonucleotide reductase that requires iron for DNA synthesis.[2]

To overcome the limitation of the CA-fluorescent assay, Hider and co-workers have recently developed a range of highly sensitive novel fluorescent iron chelators such that a fluorescent function (i.e. coumarin substitutes) is covalently linked to 3-hydroxypyridin-4-one (HPO). Among these, CP655 (7-diethylamino-N-[(5-hydroxy-6-methyl-4-oxo-1,4-dihydropyridin-3-yl)methyl]-N-methyl-2-oxo-2H-chromen-3-carboxamide), which is a moderately lipophilic fluorescent chelator, was found to be the most sensitive probe for monitoring intracellular LIP.[102,103] The concentration of the intracellular chelatable iron pool in hepatocytes was determined by this probe to be 5.4+/-1.3 micromolar. [103]

To measure the distribution of LI in subcellular compartments, Petrat and co-workers introduced a fluorescein-based iron sensor 'Phen green SK' incorporating a 1,10-Phenanthrolin chelation unit.[104,105] This iron sensor was capable of detecting much higher cytosolic LI concentrations in cultured hepatocytes as well as being able to detect LI in subcellular compartments in a number of cultured cell lines. The quantitative studies of

chelatable LI with 'Phen green SK' revealed that both lysosomal and mitochondrial compartments have 2-3 fold higher levels of LI when compared to the cytosolic pool.[105-107] These studies also highlighted the surprisingly high content of redox-active LI in the nucleus with clear implications for an increased probability of DNA damage.[37]

The presence of high levels of LI in mitochondria, lysosomes and nucleus makes these organelles highly vulnerable to oxidative damage upon exposure to oxidising agents such as UVA or H_2O_2.[26] For example we have observed that in skin fibroblasts, UVA promotes an immediate and dose-dependent damage to lysosomal, mitochondrial and nuclear membranes that can be prevented by organelle-permeable iron chelator pre-treatment.[14,26,28,87,88,108]

The development of real-time fluorimetry and flow cytometry techniques in conjunction with targeted fluorescent iron sensors capable of monitoring concurrently the cytosolic and mitochondrial LI changes[109] as well as lysosomal/endosomal-specific fluorescent iron sensors with high responsiveness towards alterations of endosomal/lysosomal LI[100] have provided valuable insights into understating the mechanisms underlying the subcellular LI distribution and trafficking under physiological and patho-physiological conditions. Below, we provide a summary of the recent findings in this domain.

2.4.1 Cytosolic LIP. The main source of cytosolic LIP is via Tf-TfR1-mediated endocytosis (see Fig. 1).[110] Cells which require iron express the TfR1 on their surface, which binds two molecules of Tf. Transferrin has a high affinity for Fe^{+3} (Kd= 10^{-23} mol/L) and its primary function is to accept iron from plasma (which then takes on the diferric form) and to transport iron into various cells and tissues, by binding to TfR1. The Tf-TfR1 complex is then internalized by receptor-mediated endocytosis, where the diferric Tf-TfR1 complex is taken into the cell.[60, 111] Once in the endosome, the pH decreases via a proton pump present on the endosomal membrane allowing the Fe^{3+} ions to dissociate from the Tf-TfR1 complex. The endosomal ferrireductase 'Steap3' (six-transmembrane epithelial antigen of the prostate-3),[112] is thought to convert Fe^{3+} to Fe^{2+} in the endosomes, allowing Fe^{2+} ions to be transported out of the endosomes by divalent metal transport protein, DMT1.[113] Once in the cell, Fe^{2+} ions are thought to enter the cytosolic LIP. A recent study by Cabantchik and co-workers using live and real-time fluorescence techniques has revealed that in K562 human erythroleukaemia cells, most of Tf-bound iron is indeed delivered to the cytosolic LIP by a saturable

mechanism that is quantitatively dependent on TfR1 levels, endosomal acidification/reduction for dislodging iron from Tf and the ensuing translocation of LI into the cytosolic compartment.[109]

The endosomes containing the Tf–TfR1 complex then undergo exocytosis to recycle TfR1 and return the apo-Tf to the bloodstream where it is able to bind more iron from the liver (see Fig. 1).[114]

The cytosolic LIP can reach several targets (see Fig. 1). It can either be stored in Ft for long term storage[115] or be readily used in the synthesis of various proteins and enzymes such as ribonucleotide reductase.[116] In general it is assumed that most of the cytosolic LI that is not metabolised is stored in Ft. Ferritin is an ubiquitously expressed cytosolic iron storage protein which forms a hetero-oligomeric protein shell composed of 24 Ft light (L-Ft, 19kD) and heavy (H-Ft, 21kD) chain subunits. Up to 4500 iron atoms can be sequestered in Ft as a crystalline core of ferric ions (Fe^{3+}). Storing iron in Ft prevents LI from generating toxic radicals and allows the regulated release of iron.[115] Studies with murine and rat models have shown that loss of cytosolic iron storage Ft by down-regulating or deleting H-Ft leads to an increase in LIP, oxidative stress, tissue damage and cell death.[117,118] Recent work has identified a cytosolic iron chaperone, PCBP1 [poly (rC)-binding protein 1] that transports iron to Ft.[119] Depletion of PCBP1 in human cells inhibited Ft iron loading and increased cytosolic LIP.[119]

The cytosolic LIP may also reach the mitochondria directly via the putative mitochondrial iron import protein, mitoferrin (Mf), for synthesis of heme and iron-sulfur clusters.[110] The latter pathway was first supported by Lange *et al* who measured Fe^{2+} ingress into isolated mitochondria.[120] The recent development of real-time fluorimetry and flow cytometry techniques in conjunction with targeted fluorescent iron sensors capable of monitoring concurrently the cytosolic and mitochondrial changes in LI evoked by Tf-bound iron[109] confirmed the initial observation by Lange *et al.*[120] Using this methodology, Cabantchik and co-workers demonstrated that the bulk of Tf-bound iron initially delivered to cells via endocytosis can be quantitatively traced in the cytosol as cytosolic LIP, from where it is distributed to other cell compartments, notably mitochondria.[109] At present it is not known whether cytosolic LI is delivered to mitochondria by high-affinity chaperone-like moieties, as proposed for Ft or simply by diffusion.[109,110,119] The mitochondrial protein frataxin, that is deficient in individuals suffering from neuromuscular disease Friedreich's ataxia (FRDA), could conceivably fulfil the chaperone-like function, as frataxin is thought to

deliver iron for the synthesis of iron- sulfur clusters to these organelles and has also been recently found in the cytosol.[110] The presence of a high-affinity chaperone-type iron acquisition system to mitochondria is also supported by a study by Garrick *et al*, who demonstrated that high affinity iron chelates could donate iron for heme synthesis.[121]

It has been proposed that iron delivery to mitochondria may also occur via an alternative mechanism that bypasses the cytosolic LIP route. This includes the direct transfer of iron from endosomes (i.e. iron sequestered by Tf-TfR1 pathway) to the mitochondria as a 'kiss-and-run' mechanism, mediated by DMT1 (as endosomal iron exporter) and Mf (as mitochondrial iron importer) (see Fig. 1).[122,123] This alternative mechanism is thought to apply primarily to erythroid cells which require a high rate of iron utilization for heme synthesis.[122-125] The terminal step of heme biosynthesis requires the insertion of Fe^{2+} ion by ferrochelatase enzyme into heme precursor, protoporphyrin IX (PPIX) to produce heme. In hemoglobin-synthesizing cells, the vast majority of iron released from endosomes must cross both the outer and the inner mitochondrial membranes to reach ferrochelatase.[126] It is remarkable that in these cells iron acquired from Tf continues to flow into mitochondria, even when the synthesis of PPIX is markedly compromised *in vitro*[127-129] (by isonicotinic acid hydrazide or succinylacetone) or *in vivo*[130] (patients with erythroid specific 5-aminolevulinate synthase deficiency). A significant proportion of non-heme iron that accumulates in mitochondria under these circumstances is in a form readily available for heme synthesis when PPIX formation is restored.[127-129] The 'kiss-and-run' is defined as a transient endosome-mitochondrion interaction to mediate iron transfer between these organelles. This model proposes that, after iron is released from Tf in the endosome, it is passed directly from endosomal iron exporter DMT1 to mitochondrial iron importer Mf until it reaches the ferrochelatase in the mitochondrion. Interestingly, when heme synthesis is inhibited in definitive erythroid cells, very little[128,129] or no[131] iron accumulates in cytosolic Ft. In contrast, it is well established that in normal non-erythroid cells, iron in excess of metabolic needs ends up in Ft. Thus, it seems highly likely that in erythroid cells the transport of iron into mitochondria is controlled differently than in non-erythroid cells.[122,126]

Metabolites can cross the mitochondrial outer membrane through a large diameter voltage dependent channel, also known as mitochondrial porin,[132] while the inner mitochondrial barrier can only be crossed with the aid of specialised mitochondrial solute carrier family (MCF), localised in the inner mitochondrial membrane.[133] Mitoferrin-1 (Mf1, Slc25a37), is a member of the MCF that functions as an essential iron importer for the

synthesis of heme and iron-sulfur clusters in erythroblasts.[124] Recent work has shown that in erythroblasts, ferrochelatase forms an oligomeric complex with Mfl and Abcb10 to synergistically integrate mitochondrial iron incorporation and use for heme biosynthesis.[125] A similar protein, named mitoferrin-2 (Mf2) has also been identified in Zebrafish and mammals that unlike erythroid Mf1, is expressed at low levels in all tissues.[134] The latter may be involved in iron import into the mitochondria of non-erythroid cells.[133] In skin fibroblasts, we have observed that growing cells under low serum concentration for several days could substantially decrease the basal intracellular level of LIP (as measured by IRP1 RNA-binding activity) and cause a related increase in the basal level concentrations of the mitochondrial heme precursor PPIX. The latter study suggests that the extent of heme biosynthesis in mitochondria of skin cells is directly related to the availability of intracellular LIP.[135]

Alternatively the cytosolic LIP may reach the plasma membrane where it is exported via the iron exporter protein FP1 (see Fig. 1).[110] The divalent iron exporter FP1 (also called IREG1 and MPT1) has been found in the basolateral membrane of enterocytes.[136] It has been shown that FP1 is only expressed in liver, spleen and kidney, so the mechanism of iron export from other cell types is unclear.[37]

2.4.2. Mitochondrial LIP. The majority of cellular iron is utilised in the mitochondria for the biosynthesis of both heme and iron-sulfur clusters.[137] This makes the mitochondrion an important organelle in iron trafficking.[138] The chemical form of available iron inside the mitochondrion remains an elusive issue.[133] Studies with improved fluorescent iron chemosensors such as Rhodamine B-based sensors selectively detected a form of chelatable LI in the mitochondrial matrix of cultured rat hepatocytes and cardiomyocytes,[139] human erythroleukemia K562 cells[107,109] and fibroblasts from FRDA patients.[140] As mitochondria are the main cellular iron consumers, as well as being the principal source of $O_2^{\cdot -}$, LI levels are kept to minimum via tight coordination of the rate of influx with the rate of incorporation into heme and iron-sulfur clusters.[110,133]

2.4.3 Lysosomal LIP. Autophagy appears to be the main process that delivers substrates to the lysosomal compartments in all cells except erythrocytes, which lack lysosomes.[141] The ongoing decomposition of iron-containing metalloproteins such as Ft and mitochondrial electron transport complexes within these acidic organelles is accompanied by

the release of redox-active LI. Studies by Petrat and co-workers suggest that the intra-lysosomal pool of LI is a major source of intracellular LIP, being 2-3 fold higher than cytosolic LIP.[106] The presence of LI in lysosomes has also been confirmed by Hider and co-workers who designed a series of HPO-based highly specific fluorescent probes that exclusively accumulate in lysosomal compartments and possess high responsiveness toward alterations of lysosomal LIP.[100]

Upon export from lysosomes, this rich source of LIP may contribute to the continued synthesis of new iron-containing proteins,[142-144] although it is not known exactly how redox-active LI is transported from lysosomes to other subcellular compartments. The transport of lysosomal LI to cytosol may involve DMT1 similar to release of iron from late endosomes, although it is not known whether iron bound to DMT1 is redox-active.[141] An alternative mechanism for the delivery of LI from late endosomes/lysosomes to mitochondria may involve the direct transport between these two organelles following temporary close contact (see 'kiss-and-run' in section **2.4.1**).[122,123]

2.4.4 Nuclear LIP. Petrat *et al* were the first to detect a source of LI in the nucleus of a series of cultured cell lines.[105-107] Cabantchik and co-workers further confirmed the presence of LI in nucleus of cardiac cells by means of a fluorescent LI sensor (i.e. CAL-G) covalently attached to histones.[145] This study also highlighted the existence of a dynamic and rapid equilibrium between the cytosolic and nuclear LI pools presumably via nuclear pores.[145]

2.5 The Role of Subcellular LIP Distribution in the Cellular Response to Oxidative Stress

Earlier studies aimed at understanding the role of intracellular LIP in potentiating oxidative damage in cells consisted of loading the cells with Tf-Fe or iron-salts followed by an oxidative challenge of cells with oxidizing agents such as t-butyl-hydroperoxide (TBHP), H_2O_2 or UVA radiation. These studies indicated that concomitantly with the rising of LIP levels in the cytosol (as measured by CA-assay), there was demonstrable rise in ROS production (possibly via Fenton reaction), lipid peroxidation and eventual cell death.[2,146,147] Recent studies revealed however that the relationship between cytosolic LIP and intracellular ROS generation is more complex than previously thought. Indeed it is now known that

intracellular LIP can not only act as a potent generator of ROS but its level can also be raised by oxidants or reductants.

2.5.1 The Role of Ft Iron in Oxidant-mediated Increase in Cytosolic LIP. The mechanism of iron release from Ft is still under investigation. Various reductants and chelators, including physiological and toxicological substances are capable of releasing iron from Ft.[37] It has been shown that under conditions of iron depletion (e.g. after iron chelation treatment) and in absence of extracellular iron resources, Ft iron can be directly released in the cytosol via proteolysis to replenish the cytosolic LIP.[148] Breuer *et al* studied the possible alteration of intracellular LIP in K562 cells that were treated with TBHP or H_2O_2.[149] The CA-assay revealed that a short exposure to these oxidants can raise the cytosolic LIP in a dose-dependent manner.[149] This phenomenon was attributed to reductive release of iron from Ft,[149] as has been previously shown *in vitro* for superoxide radicals,[150-152] sulfhydryl reagents[153] and other agents including UVA light.[154-157]

Direct measurements of intracellular LIP by electron paramagnetic resonance also indicated an expansion in intracellular LI with a concomitant drop in intracellular Ft levels in the liver of rats subjected to oxidative stress in the form of phorone (a GSH-depleting drug) or ischemia-reperfusion.[57,86] The early drop in Ft in the latter studies was demonstrated to be a proteolytic degradation event leading to an increase in intracellular LIP.[57,86] Studies from this laboratory have also demonstrated that the UVA-induced increase in cytosolic LIP is partly due to proteolytic degradation of Ft.[14]

Ferritin degradation in cytosol may occur via proteasomes upon oxidative damage, pathological conditions, FP1 overexpression or treatment with oral iron chelators such as deferiprone.[115] However lysosomal proteases have also been shown to be involved in the oxidant-mediated degradation of Ft and the related increase in cytosolic LIP. Under physiological conditions, the predominant mechanism of iron release from Ft is through the constitutive degradation of the protein in lysosomes by specific lysosomal proteases, notably chymotrypsin.[14] The half-life of Ft in the cytosol is estimated to be 24-72h (depending on the cell type), after which it is transported into lysosomes where its iron is thought to be recycled for cellular requirements notably heme synthesis.[141,158] The high turnover of both endocytosed and autophagocytosed iron-containing macromolecules (e.g. Ft and mitochondrial electron transport complexes) cause the accumulation of the potentially

hazardous LI in lysosomal compartments that render these organelles highly vulnerable to oxidative damage upon exposure to oxidizing agents including UVA radiation.[26] In skin fibroblasts, UVA has been shown to promote the proteolytic release of Ft iron in the cytosol. The UVA-induced proteolytic degradation of Ft occurs as a result of radiation-mediated damage to lysosomal membranes leading to leakage of lysosomal proteases to the cytosol.[14] Pretreatment of skin fibroblasts with Leupeptin and Chymostatin (lysosomal protease inhibitors) prevented the UVA-induced Ft degradation and caused a substantial decrease in the level of cytosolic LI released by UVA.[14] We have also previously reported a clear link between the lower basal level of Ft and UVA-induced release of cytosolic LI in keratinocytes that may be in part responsible for the resistance of primary skin keratinocyte cells to UVA-induced LI damage as compared to their UVA-sensitive matched primary skin fibroblasts that contain higher basal Ft level and release more cytosolic LI upon UVA exposure.[28]

So it appears that Ft iron can replenish the cytosolic LIP upon oxidative stress as it can be released either reductively or as a result of its proteolytic degradation.[110] Therefore Ft plays a dual role in LIP homeostasis, acting on the one hand as an iron-sequestering protein and on the other hand as a potential source of LIP.[26,37]

2.5.2. The Role of Lysosomal LIP and Proteases in Oxidant-mediated Cell Damage. The oxidant-mediated destabilization of lysosomal membranes and rapid leakage of both lysosomal LIP and proteases to the cytosol leads to a cascade of events resulting in cell death (i.e. apoptotic or necrotic cell death, depending on the extent of insult).[14,26,27,141]

The role of redox-active LI in compromising the stability of lysosomes under oxidative stress is well-demonstrated in studies involving simultaneous exposure of cells to an oxidising agent and a potent iron-specific chelator. For example Brunk and co-workers have reported that the combined treatment of cells with H_2O_2 and the highly lipophilic iron chelator salicaldehyde isonicotinoyl hydrazone (SIH) almost fully prevents both lysosomal rupture and cell death. The lack of oxidative effect of H_2O_2 in presence of SIH strongly suggests that H_2O_2 *per se* is not particularly toxic but rather must work in concert with iron in order to damage cells.[141] Similarly the pre-treatment of skin cells with lysosomotrophic strong iron chelator desferrioxamine (DFO) or the highly lipophilic iron chelator pyridoxal isonicotinoyl hydrazone (PIH) significantly protected the cells against UVA-mediated lysosomal damage (unpublished data, this laboratory) and the ensuing necrotic cell death[87] by

virtue of their strong iron-chelating properties.[141,159,160] It is noteworthy that H_2O_2 and O_2^1 are recognised as the predominant intracellular ROS generated by UVA promoting biological damage in exposed tissues via iron-catalysed oxidative reactions.[17-20]

It has been suggested that the concomitant release of lysosomal proteases and LIP following oxidative damage and the consequent destabilisation of the lysosomal membranes might activate feedback processes that would cause further lysosomal rupture. Such feedback processes may be either LIP-mediated and/or due to activation of lytic cytosolic pro-enzymes such as caspases.[14,161,162] Lysosomal proteases such as cathepsins B/L/D and chymotrypsin have been specifically detected in the cytosol of cells treated with oxidising agents, indicating their translocation from lysosomal compartments to the cytosol as a result of oxidative damage to lysosomal organelles.[14,27,161] The level of chymotrypsin, the lysosomal protease responsible for the degradation of Ft, was found to increase in a dose-dependent manner in the cytosol of skin fibroblasts subjected to a series of doses of UVA at natural exposure levels.[14] The latter provided a rational explanation for the observed rapid proteolytic degradation of Ft in the cytosol of skin cells subjected to UVA radiation.[14] Iron loading of cells prior to UVA radiation exacerbated both the lysosomal damage (unpublished data, this laboratory) and the ensuing cell death in skin cells.[28]

Lysosomal organelle rupture resulting from intra-lysosomal Fenton-type reactions upon exposure to oxidative agents has also been associated with enhanced DNA damage due to sudden release of lysosomal LI into the cytoplasm.[162] It has also been suggested that permeabilization of mitochondrial membranes by release of lysosomal proteases may also induce a loss of mitochondrial membrane potential, irreversibly leading to cell death.[100,142,163] The pre-treatment of cells with lysosomotrophic iron chelator DFO prevented the oxidant-induced lysosomal membrane damage, DNA damage, loss of mitochondrial membrane potential and cell death,[26,164,165] implying the important role of lysosomal LI in catalysing harmful oxidative damage.

2.5.3 The Role of Heme iron in Oxidant-mediated Increase in Cytosolic LIP. In addition to Ft iron, heme iron may also contribute to the oxidant-mediated rise in cytosolic LIP. Studies from Tyrrell's laboratory have shown that immediately after exposure of skin fibroblasts to UVA or H_2O_2, heme is released from microsomal hemoproteins to the cytosol.[15] Although heme itself is not a source of LIP, it is a substrate for heme-catabolising

enzyme HO which could release the heme iron and expand the pool of potentially harmful cytosolic LI. The role of heme iron in exacerbating the UVA-induced oxidative damage was well illustrated by Kvam *et al* who showed that overexpression of HO-2 in a Hela cell line could cause a transient hypersensitivity to UVA; this effect depended on release of iron from heme.[93] Hemin treatment was also found to strongly sensitise the skin keratinocytes to UVA-induced necrotic cell death.[28] The latter study indicated a clear link between heme iron, Ft iron and the cytosolic LIP i.e. overnight hemin treatment induced a 4-fold increase in Ft levels that contributed to a 3-4 fold increase in cytosolic LIP in keratinocytes following UVA radiation.[28] These studies highlighted the pro-oxidant potential of Ft iron in exacerbating the UVA-induced oxidative damage, as its upregulation led to enhanced cellular damage and cell death. This is in stark contrast to studies showing that upregulation of Ft (i.e. by overexpression of H-Ft or hemin treatment), could reduce oxidative stress responses and protect the cells against H_2O_2-induced cyto- and geno-toxicity.[96,166,167] The differential mechanism of LI-induced oxidative-damage induced by H_2O_2 and UVA may be the reason for this discrepancy.

2.5.4 The Role of Mitochondrial LIP in Oxidant-mediated Cell Damage and Death. In addition to cytosolic and lysosomal LI, mitochondria have also been recognised as the principal destination of LI in cells and therefore a primary site of pro-oxidant generation rendering these organelles particularly susceptible to oxidative damage. Since mitochondria are the major sites of oxygen consumption and LI is also a potent inducer of ROS formation, the simultaneous presence of oxygen and iron appears to be detrimental to the organelles. Chelatable LI was detectable in the mitochondria of cultured hepatocytes and cardiomyocytes with high affinity probes[139] and with iron chelators capable of inhibiting mitochondrial ROS generation.[168] Although mitochondria have evolved mechanisms to regulate iron-dependent damage and maintain mitochondrial functionality, it appears that the organelles remain susceptible to oxidative damage and alterations in mitochondrial iron homeostasis ultimately lead to pathological phenotypes and cell death.[26,28]

The consequences of a pathological rise in mitochondrial LIP have been well documented in a series of genetic disorders with defective cellular iron utilization (i.e. trafficking and incorporation into proteins) whereby LI concentrations rise to toxic levels in mitochondria of excitable cells often leaving the cytosol iron-depleted. These inherited disorders include FRDA, myopathy with iron-sulfur cluster scaffold protein deficiency, X-

linked sideroblastic anemias and neurodegeneration with brain iron accumulation.[169] The studies in this field have highlighted a positive correlation between preferential accumulation of redox-active LI in mitochondria and oxidative damage in sensory neurons, the myocardium and endocrine glands.[169] Wong *et al* have also demonstrated that cultured fibroblasts from FRDA patients that contain high mitochondrial iron level are highly sensitive to iron stress and significantly more sensitive to H_2O_2-mediated cell death than controls.[170]

In addition to its involvement in pathological conditions, mitochondrial LI level has also been recently identified as the key determinant of susceptibility of cells to oxidative stress. Studies from this laboratory have demonstrated that exposure of cultured human skin fibroblasts to UVA promotes immediate damage to mitochondrial membranes and that this plays a key role in UVA-induced necrotic cell death.[28] The mitochondrial damage leads to abrupt interruption of electron chain reactions within the mitochondrial membrane and production of ROS as well as depletion of mitochondrial ATP that leads to necrotic cell death of UVA-irradiated skin cells.[28] Pretreatment of skin cells with highly lipophilic iron chelators SIH, PIH and their caged-photolabile derivative prevented damage to mitochondrial membranes, ATP depletion and the ensuing necrotic cell death, as these compounds were capable of accessing and sequestering the loosely available LI in these organelles.[26,87] Similarly Wong *et al* observed that FRDA fibroblasts treated with DFO were rescued from H_2O_2-induced death to a greater extent than controls.[170] Chelating of intracellular iron with the iron chelator DFO has also provided some protection against the formation of H_2O_2-induced mitochondrial DNA breaks.[171] Richardson and co-workers have also demonstrated that the mitochondria permeable iron chelator 2-pyridylcarboxaldehyde 2-thiophenecarboxyl hydrazone (PCTH) was more effective than DFO in preventing the H_2O_2-induced cytotoxicity in cultured FRDA fibroblasts.[172]

The critical role of lysosomal and mitochondrial LI in oxidative injury has also been well demonstrated in a series of studies by Lemasters and co-workers showing that translocation of LI from lysosomes into mitochondria is a key event during oxidative stress-induced hepatocellular injury and involves the synergistic action of oxidative stress and LI to promote toxic radical formation, mitochondrial dysfunction and cell death.[173]

2.5.5 The role of Nuclear LIP in Oxidant-mediated Iron Damage. The presence of high amount of LI in the nucleus is thought to make these organelles highly susceptible to

oxidative damage. A study from this laboratory has shown that UVA is capable of promoting LI-mediated oxidative damage to nuclear membrane of skin fibroblasts leading to a transient increase in permeability of the nuclear membrane to proteins.[108] The pre-treatment of fibroblasts with iron chelators prevented the UVA-induced destabilisation of nuclear membrane.[108] Furthermore it was found that the slow kinetics of induction of Nuclear Factor-kappa B (NF-kappaB) by UVA relative to other oxidants is due to a transient increase in permeability of the nuclear membrane to proteins and occurs as a result of LI-mediated damage to nuclear membrane.[108] The apparent slow response of NF-kappaB to UVA radiation is likely to have consequences on the kinetics of activation of NF-kappaB target genes in the nucleus notably pro-inflammatory cytokines and proto-oncogenes.[108,174]

The nuclear redox-active LI is also thought to be involved in DNA damage induced by H_2O_2 and other oxidising compounds.[37] This assumption is strengthened by studies showing a significant correlation between cellular LIP and the yield of 7,8-dihydro-8-oxoguanine base damage (i.e. a typical marker of ROS-mediated DNA damage) and DNA breaks in cells treated with oxidising agents such as H_2O_2.[37] Furthermore pre-treatment of cells with the iron chelator DFO has been shown to prevent the formation of DNA breaks and cytotoxicity by H_2O_2 to nuclear DNA.[37] Interestingly UVA doses that promote a substantial increase in cytosolic LIP, have also been shown to induce 7,8-dihydro-8-oxoguanine base damage in human skin fibroblasts, a classical marker of oxidised DNA.[175]

3 UVA-Induced Iron-Mediated Damage

3.1 Mechanisms Underlying the Phenomenon of UVA-induced LI Release

Fig. 2 summarises potential pathways by which UVA promotes an increase in cytosolic LIP. Our research aimed at understanding the source of UVA-induced increase in cytosolic LIP has revealed that preventing UVA-induced Ft degradation with specific protease inhibitors only partially decreased the observed increase in cytosolic LI following UVA radiation.[14] The latter strongly suggested that other sources in addition to Ft's iron contribute to UVA-induced immediate increase in cytosolic LIP.

Clearly the presence of high level of redox active LI in lysosomes, mitochondria and nucleus of skin cells sensitises these organelles to UVA-induced oxidative damage, so it is not surprising that exposure of skin cells to UVA at natural exposure levels promotes dose-dependent damage to these organelles.[28,88,108] The rapid release of LI from these organelles is

likely to contribute to the measurable increase in cytosolic LIP which in turn will exacerbate the oxidative damage to cell constituents. While the cytosolic LIP is physiologically kept low in cells by storage in Ft, the UVA-induced degradation of Ft by lysosomal proteases, further contribute to the expansion of cytosolic LIP. The lack of the cytosolic iron storage protein, Ft within the critical first hours after irradiation further exacerbates the iron-catalyzed damage in irradiated skin cells, since a potentially harmful excess of cytosolic LI can not be safely sequestered. Indeed increase in cytosolic LI along with ROS generated by UVA radiation promotes further peroxidative damage in exposed skin cells notably in plasma membranes and this results in turn in a loss of cell membrane integrity. The latter predisposes the cells to necrotic cell death as a result of the influx of extracellular media to the intracellular environment leading to swelling and rupture of subcellular compartments and cell lysis.[26]

Concomitant with destabilisation of cell membrane, UVA also promotes an immediate depletion in mitochondrial ATP, that is a hallmark of necrotic cell death.[28,87] The UVA-induced mitochondrial ATP depletion is thought to be triggered by several simultaneous events. Firstly the presence of a high level of LI in mitochondria may initiate the destabilisation of mitochondrial membranes upon UVA treatment as a result of LI-induced oxidative damage. Secondly the UVA-induced expansion of cytosolic LI by lysosomal and nuclear damage as well as Ft degradation may exacerbate the iron-catalysed oxidative damage that already occurs in the mitochondrial membrane. Finally the rapid release of lysosomal proteases after UVA radiation might further attack the mitochondrial membrane leading to additional permeabilisation of the mitochondrial organelles. All these events will almost certainly contribute to the observed UVA-induced rapid depletion of mitochondrial ATP that leads to the demise of cells in the form of necrosis. Replenishing the cellular ATP with glucose led to a substantial decrease in the extent of UVA-induced necrotic cell death in skin fibroblasts.[28] Most importantly pre-treatment of skin fibroblasts with iron chelators dramatically decreased the level of mitochondrial ATP depletion, plasma membrane damage and necrotic cell death.[28,87]

Previous observations from this laboratory have shown that although UVA promotes mitochondrial damage and cytochrome c release in fibroblast cell lines such as FEK4, these cell lines are particularly resistant to UVA-induced apoptotic cell death.[27] As the presence of ATP is essential for the activation of apoptosis protease activating factor-1 (Apaf-1) and subsequent activation of caspases that induce apoptosis[27], it appears that the UVA-induced

immediate depletion of ATP in skin fibroblasts provides a rational explanation for the predominance of necrotic cell death induced by UVA.

In all these damaging processes, LI is clearly involved as pre-treatment of cells with both the lysosomotropic iron chelator DFO and the highly lipophilic/cell organelle-permeable chelators SIH and PIH fully protected the skin cells against UVA-induced lysosomal, mitochondrial and nuclear membrane damage.[28,87,108] In contrast pre-treatment of skin cells with membrane antioxidants such as α-tocopherol succinate (α-Toco), Trolox and butyryl hydroxytoluene (BHT) only partially protected the cells against UVA-induced peroxidative damage presumably because of the high amount of LI still present in these organelles. Indeed the CA-assay revealed that UVA still promotes an increase in LIP in cytosolic compartments of cells pre-treated with α-Toco or BHT (unpublished data, this laboratory). Numerous other studies in this field have also demonstrated that 'conventional' antioxidants have had a very modest protective effect, presumably because the investigators have overlooked the phenomenon of UVA-induced excess LI release in cells that could still contribute to generation of ROS and oxidative damage.[26] Conventional radical scavengers can often not neutralise the overall ROS production upon exposure of cells to strong oxidising agents such as UVA which also promote redox-active LI release in cells. Because of this dual action, only bifunctional antioxidants targeting both LI and ROS have been effective in protecting skin cells against UVA-induced oxidative damage and cell death.[26,88,176,177]

UVA not only promotes the early degradation of Ft, but also the degradation of a series of hemoproteins notably catalase and microsomal hemoproteins. Microsomal hemoproteins are found to be immediately degraded in human skin fibroblasts following relatively low doses of UVA with the consequent release of free heme.[15,16] The UVA-induced heme release provides another likely source of LI that may contribute to UVA-induced increase in cytosolic LIP, as it has been shown that it can be readily broken down to release LI by both the constitutive HO-2 as well as the inducible HO-1 enzymes. The UVA-induced heme release has been identified as a key factor in the upregulation of the HO-1 gene in skin fibroblasts.[16]

3.2 Mechanisms Underlying the Resistance of Keratinocytes to UVA Radiation

Compared to skin fibroblasts, skin keratinocytes are more resistant to UVA-mediated membrane damage[178] and cytotoxicity,[13] although the underlying mechanism is yet to be

determined. The evaluation of intracellular LIP in a series of matched human primary skin fibroblasts and keratinocytes revealed that the basal level concentrations of both cytosolic LIP and Ft in keratinocytes is several-fold lower than in fibroblasts.[28] Keratinocytes also possess less lysosomal organelles than fibroblasts. The quantification of lysosomal membrane damage by neutral red dye uptake assay revealed that the overall UVA-induced damage to lysosomal organelles in keratinocytes is also much lower than in skin fibroblasts (unpublished data, this laboratory). Moreover, the quantification of lysosomal cysteine proteases such as cathepsin-B, -L and -D with ELISA assays revealed that keratinocytes possess up to 20-fold lower cathepsin levels than skin fibroblasts. Accordingly following UVA irradiation, the level of lysosomal cathepsins delocalised to cytosol was found to be much higher in skin fibroblasts than keratinocytes. This was also confirmed by *in situ* detection of cathepsin-B release by epifluorescence microscopy after immunostaining of cells with a polyclonal rabbit anti-human cathepsin-B antibody (unpublished data, this laboratory).

So it appears that although UVA triggers lysosomal damage, Ft degradation and cytosolic LI release in keratinocytes, the absolute level of UVA-induced LI release remains several-fold lower in these cells than in fibroblasts and this almost certainly contributes to the lower level of mitochondrial damage, ATP depletion and necrotic cell death following radiation treatment.[28] We propose that a combination of low basal and UVA-induced increase in cytosolic LI, low basal level of Ft and low lysosomal organelle and low lysosomal cathepsin content will all contribute to the higher resistance of keratinocytes to UVA-induced membrane damage and cell death. The level of lactate dehydrogenase release from UVA-irradiated keratinocytes was found to be 4-fold lower than from UVA-irradiated fibroblasts, consistent with the notion that keratinocytes are more resistant to UVA-induced membrane damage (unpublished data, this laboratory). Furthermore the UVA dose to give an equivalent level of necrosis was found to be several-fold lower in fibroblasts than in keratinocytes.[28] An artificial increase in intracellular Ft levels in keratinocytes by overnight treatment of cells with hemin or iron-citrate caused a massive increase in cytosolic LI following UVA and a strong sensitisation of the cells to very low doses of UVA, implying the importance of cytosolic LI concentrations in increased susceptibility of skin cells to UVA-induced oxidative damage and cell death.[28,87]

Given that the upper layer of skin (epidermis) receives high levels of UV radiation on sunlight exposure, it appears that nature has considerably protected the keratinocytes against UVA-induced LI damage. Interestingly in keratinocytes HO-1 is not inducible by UVA,

however these cells exhibit a high level of constitutive HO-2 protein. In addition to HO-2, keratinocytes also possess other strong constitutive antioxidant defense molecules and enzymes[7,52] that together should be effective in protecting keratinocytes against the deleterious effect of UVA-induced oxidative iron damage, as these cells release very low amount of cytosolic LI after UVA irradiation.[28]

3.3 The Long Term Effects of UVA-induced LI Release

The kinetics of LI mobilisation in both fibroblast and keratinocyte cell lines revealed that in both cell types, the immediate increase in cytosolic LIP is sustained up to 2 h after irradiation and returns to around control value 4-6 h after UVA. The cytosolic LIP remains then unchanged as monitored up to 48 h after UVA irradiation.[28] The recovery of cytosolic LIP after UVA coincides with the reciprocal *de novo* synthesis of degraded Ft that occurs within the first 2 h after irradiation. The full recovery of Ft content to control levels was observed between 4-6 h after irradiation.[28] The kinetics of Ft synthesis after UVA irradiation further revealed that the rate of Ft synthesis increases 8-10 h after irradiation and as a result the level of cytosolic Ft becomes 2-fold higher 24 h after irradiation when compared to unirradiated control cells.[14] This phenomenon was previously thought to occur solely as a result of HO-1 induction by UVA.[94,95] However in the light of our recent findings, it appears that the UVA-induced long-term increase in Ft is a general response of UVA-irradiated cells to UVA-induced increase in cytosolic LI, where HO-1 also appears to be involved as a potential enhancer of cytosolic LI. This argument is strengthened by the observations that pre-treatment of skin fibroblasts with DFO not only blocked the UVA-induced overnight increase in Ft attributed to HO-1 induction[94] but also the UVA-induced increase in cytosolic LI.[26,28]

3.4 Concluding Remarks

Overall, our findings demonstrate that UVA-mediated increase in cytosolic LI and heme release plays a key role in the increased susceptibility of cells to UVA-induced damage and necrotic cell death. Although cells have mechanisms to remove iron (i.e. long-term increase in Ft) and heme (HO-1 activation), these defence mechanisms develop over several hours and days. Therefore it appears that high intensity short-term exposures to UVA radiation are the most likely to be damaging. This is precisely the situation for exposure to sunlamps where people tend to expose themselves habitually for short high intensity periods. A clear role for

iron in exacerbating UVA damage suggests potential pathways for protection through iron chelation. However prolonged exposure to strong iron chelators leads to severe side-effects as a result of the removal of essential iron from various iron containing proteins, including IRPs and hypoxia-inducing factor-1.[26,179,180] As a result, topical formulations of pro-chelators that are either activated by UVA light[87] or UVA-induced ROS[179] should provide a safer and more efficient alternative for skin photoprotection.[26]

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List of abbreviations

α-Toco	alpha-Tocopherol
AD	Alzheimer's disease
Apaf-1	Apoptosis protease activating factor 1
BHT	Butyryl hydroxytoluene
CA	Calcein
CP655	7-diethylamino-N-[(5-hydroxy-6-methyl-4-oxo-1,4-dihydropyridin-3-yl)methyl]-N-methyl-2-oxo-2H-chromen-3-carboxamide
DFO	Desferrioxamine
DMT1	Divalent metal transporter 1
Fe^{2+}	Ferrous ion
Fe^{3+}	Ferric ion
FP1	Ferroportin 1
FRDA	Friedreich's ataxia
Ft	Ferritin
g	Gram
GSH	Glutathione

GPx	Glutathione Peroxidase
h	Hour/hours
H-Ft	Heavy chain of ferritin
H_2O_2	Hydrogen peroxide
HO	Heme oxygenase
HO-1	Heme oxygenase 1
HO-2	Heme oxygenase 2
HPO	3-hydroxypyridin-4-one
IRE	Iron responsive element(s)
IRP1	Iron regulatory protein 1
IRP2	Iron regulatory protein 2
IRPs	Iron regulatory proteins
L-Ft	Light chain of ferritin
LI	Labile iron
LIP	Labile iron pool
mg	Milligram
MCF	Mitochondrial solute carrier family
Mf	Mitoferrin
Mf1	Mitoferrin 1
Mf2	Mitoferrin 2
NF-kappaB	Nuclear factor kappa-B
$^1O_2$	Singlet oxygen
$O_2^{\cdot -}$	Superoxide anion
$OH^{\cdot}$	Hydroxyl radical
PCBP1	poly(rC)-binding protein 1
PCTH	2-pyridylcarboxaldehyde 2-thiophenecarboxyl hydrazone
PIH	Pyridoxal isonicotinoyl hydrazone
PPIX	Protoporphyrin IX
ROS	Reactive oxygen species
SIH	Salicaldehyde isonicotinoyl hydrazone
SOD	Superoxide dismutase
Steap3	Six-transmembrane epithelial antigen of the prostate 3
TBHP	t-butyl-hydroperoxide
Tf	Transferrin
TfR1	Transferrin receptor 1

UV	Ultraviolet
UVA	Ultraviolet A
UVB	Ultraviolet B

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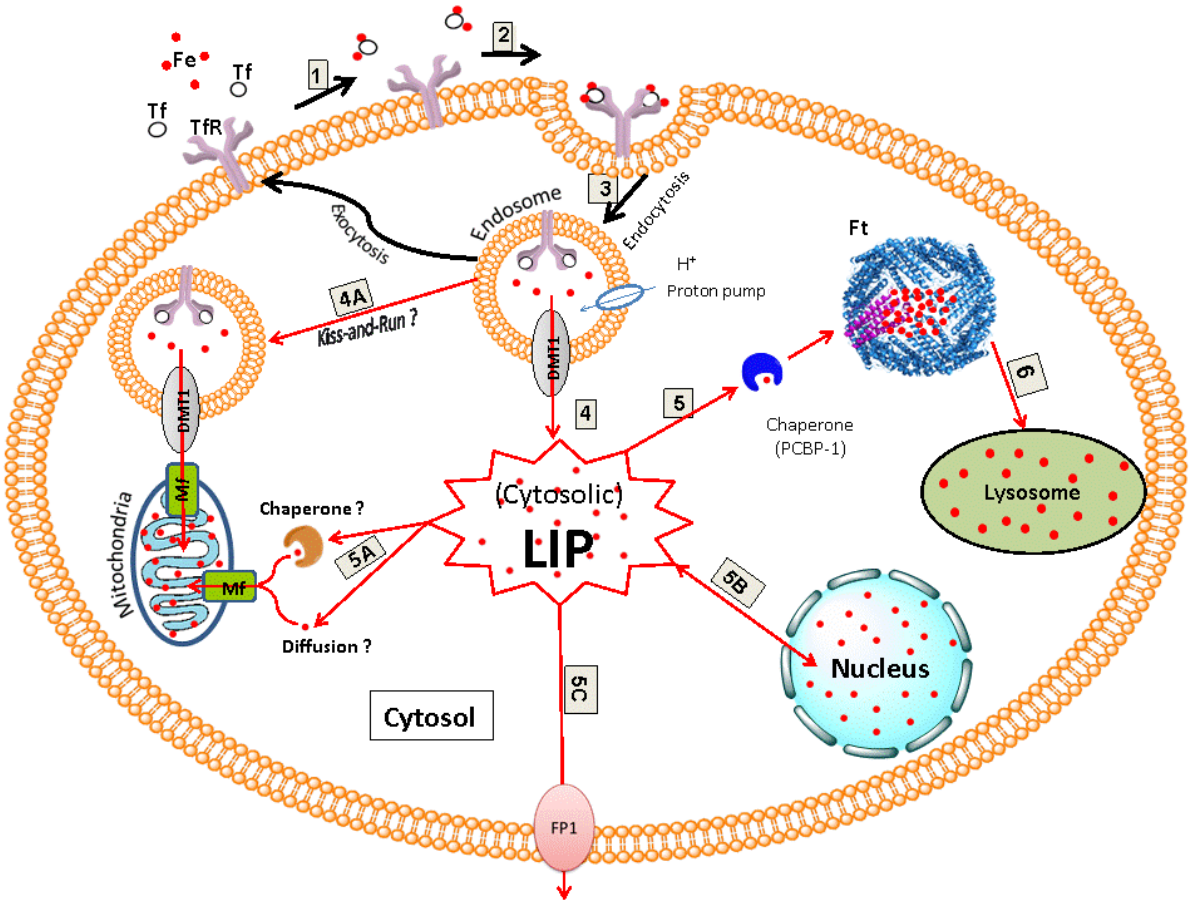

Fig. 1: Schematic diagram illustrating the pathways of cytosolic LI uptake and distribution. (Modified from Breuer *et al*, 2008)[110]. The main source of cytosolic LIP uptake is via receptor-mediated endocytosis (**1-3**)**:** In extracellular media, transferrin (Tf) binds two atoms of Fe^{3+} with high affinity (**1**). Two molecules of diferric-Tf bind to the transferrin receptor 1 (TfR1) on the cell surface (**2**). The Tf-TfR1 complex formed is internalized into an endosome. Within the endosome, iron is released from Tf following the decrease in intra-vesicular pH (**3**). Iron transfers from Tf to divalent metal transporter (DMT1) and is released in the Fe^{2+} form to the cytosol to join the pool of available cytosolic labile iron (LIP) (**4**). Endosomal iron may be directly delivered to mitochondria (i.e. without entering the cytosolic pool) via a hypothetical kiss-and-run mechanism involving DMT1 and mitoferrin (Mf) (**4A**). The cytosolic LIP can be distributed to various cellular targets (**5-6**): Cytosolic LI can be transported by a chaperone (PCBP-1) to be stored in ferritin (Ft) (**5**). Alternatively cytosolic LI may reach and replenish mitochondrial LI either via a hypothetical chaperone or possibly by diffusion (**5A**). Cytosolic LI can also establish a dynamic equilibrium with nuclear LI presumably via nuclear pores (**5B**). Cytosolic LI may also be exported from the cells via ferroportin-1 (FP1) (**5C**). The cytosolic iron stored in Ft may replenish the lysosomal LI via autophagy and proteolytic degradation of Ft in lysosomes (**6**).

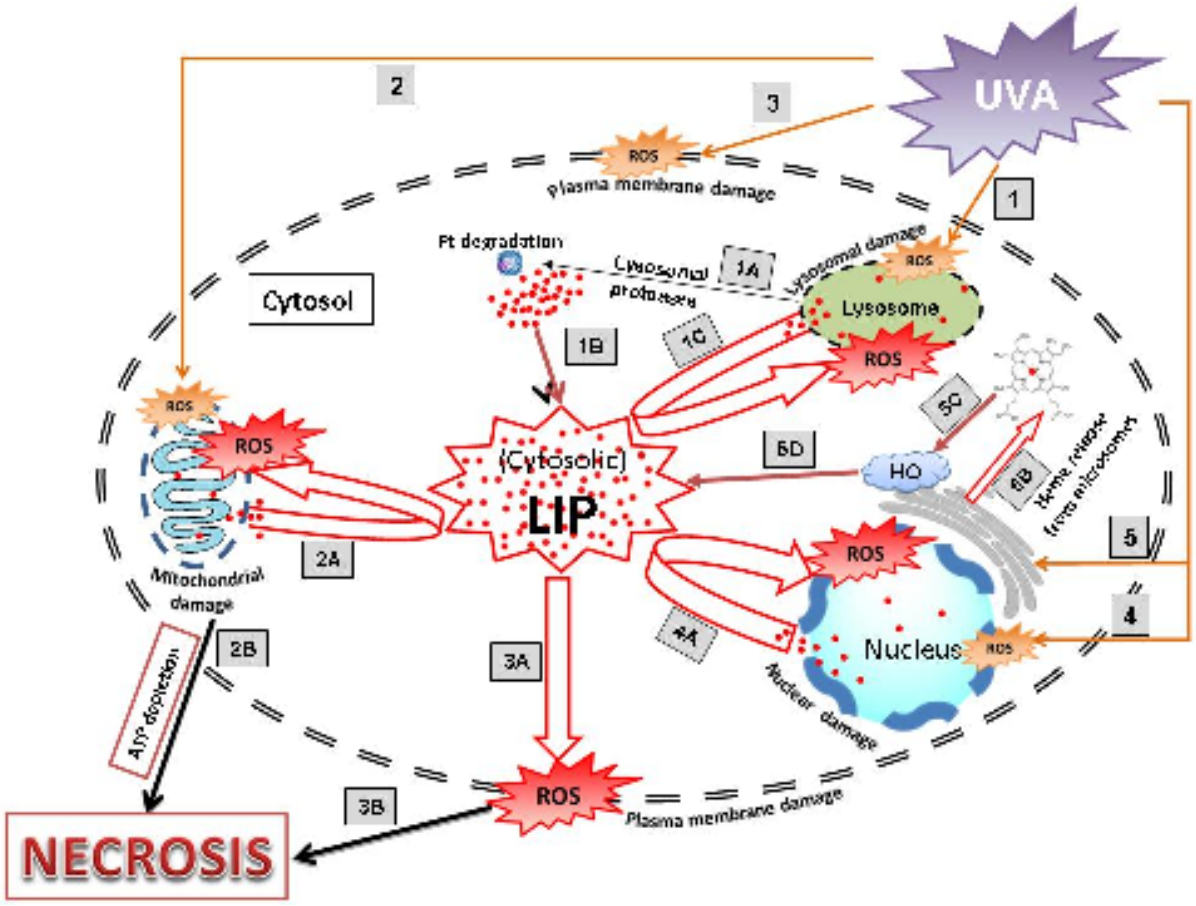

Fig. 2: Schematic diagram illustrating the pathways involved in UVA-induced cytosolic LI release and necrotic cell death in human skin fibroblasts. Exposure of skin fibroblasts to UVA generates ROS (orange colour) that promote oxidative damage in lysosomal (**1**), mitochondrial (**2**), plasma (**3**) and nuclear (**4**) membranes. UVA also promotes the immediate degradation of microsomal hemoproteins (**5**) that leads to release of free heme (**5B**) leading to increase in cytosolic LI via heme oxygenase (HO)-mediated breakdown of heme (**5C-5D**). Damage to lysosomal membrane (**1**) leads to release of lysosomal proteases (**1A**) which in turn degrade the cytosolic iron storage protein ferritin (Ft) and release its iron in the labile form (**1B**). Damage to mitochondrial membrane (**2**) leads to interruption of electron chain transport in mitochondrial membrane causing the generation of ROS, loss of the electrochemical gradient across the inner membrane and ATP depletion (**2B**). The release of potentially harmful LI in cytosol via routes (**1-5**), along with the pre-existing pool of cytosolic LI contribute to a massive increase in cytosolic pool of LI (**LIP**) that catalyses the formation of more harmful ROS (in red) that is thought to further exacerbate the peroxidative damage in the lysosomal (**1C**), mitochondrial (**2A**), plasma (**3A**) and nuclear (**4A**) membranes leading to the loss of organelles' and plasma membrane's integrity. The loss of plasma membrane integrity (**3A**) together with mitochondrial ATP depletion (**2B**) results in necrotic cell death.